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Phylogenetic relationships of bluetongue viruses based on gene S7

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Abstract

Previous phylogenetic analyses based on bluetongue virus (BTV) gene segment L3, which encodes the inner core protein, VP3, indicated a geographical distribution of different genotypes. The inner core protein, VP7, of BTV has been identified as a viral attachment protein for insect cell infection. Because the inner core proteins are involved with infectivity of insect cells, we hypothesized that certain VP7 protein sequences are preferred by the insect vector species present in specific geographic locations. We compared the gene segment S7, which encodes VP7, from 39 strains of BTV isolated from Central America, the Caribbean Basin, the United States, South Africa and Australia. For comparison, the S7 sequences from strains of the related orbiviruses, epizootic hemorrhagic disease virus (EHDV) and African horse sickness virus (AHSV) were included. The S7 gene was highly conserved among BTV strains and fairly conserved among the other orbiviruses examined. VP7 sequence alignment suggests that the BTV receptor-binding site in the insect is also conserved. Phylogenetic analyses revealed that the BTV S7 nucleotide sequences do not unequivocally display geographic distribution. The BTV strains can be separated into five clades based on the deduced VP7 amino acid sequence alignment and phylogeny but evidence for preferential selection by available gnat species for a particular VP7 clade is inconclusive. Differences between clades indicate allowable variation of the VP7 binding protein.

Keywords: Phylogenetic relationships; Bluetongue viruses; Gene S7

1. Introduction

Bluetongue virus (BTV) is an economically important insect-transmitted orbivirus (Family: Reoviridae) that causes disease in domestic and wild

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ruminants. Other closely-related orbiviruses are epizootic hemorrhagic disease virus (EHDV) of deer and African horse sickness virus (AHSV). Biting gnats of the *Culicoides* species transmit these viruses. The primary insect vector species for BTV in North America is *Culicoides sonorensis* (Tabachnick, 1996; Holbrook et al., 2000); whereas, *Culicoides insignus* is considered the common vector in Central America and the Caribbean Basin (Mo et al., 1994). Although there are a number of *Culicoides* species in South Africa the only proven vector species are *Culicoides bolitinos* and *Culicoides imicola* (Venter et al., 1998). In Australia, *Culicoides brevitarsis*, *Culicoides wadai*, *Culicoides fulvus*, and *Culicoides actoni* have been associated with BTV transmission (St. George, 1985; Standfast and Muller, 1989). Different serotypes of BTV are associated with different geographic regions. In the United States, five BTV serotypes (2, 10, 11, 13 and 17) have been isolated; whereas, serotypes 1, 3, 4, 6, 8, 12 and 17 have been isolated in Central America and the Caribbean Basin (Mo et al., 1994). Serotype 17 is the only serotype common to the US and the Caribbean Basin with serotypes 1, 3 and 4 established both in South Africa and the Caribbean Basin. Bluetongue virus serotype 2 has been isolated in South Africa and the Southeastern United States but not in the Caribbean Basin. The presence of different serotypes and vector species in distinct geographic areas suggests there may be a relationship between virus strains and competent insect vector species.

Investigations of the molecular interactions of these viruses with their insect vector hosts have shown that VP7 is involved with binding to insect membrane proteins (Xu et al., 1997). Although VP7 is an inner core protein, it is accessible on the outer surface of intact virus particles (Lewis and Grubman, 1990; Eaton et al., 1991). It has been demonstrated that BTV core particles with an exposed inner shell, consisting of VP3 and VP7, are as infectious to vector insects as intact virus particles with an outer capsid containing VP2 and VP5 (Mertens et al., 1996). In mammalian cells, however, core particles that lack VP2 and VP5 have reduced infectivity compared to intact viruses (Mertens et al., 1996). This phenomenon indicates that VP3 and VP7 are important in the ability of BTV to infect the insect vector. Both of these proteins are relatively conserved, and VP3 has distinct genotypes in different geographical areas (Pritchard et al., 1995). This study examines whether the S7 gene that encodes VP7 also displays distinct genotypes in different geographical areas that could be related to vector competence.

2. Materials and methods

2.1. Viruses and purification of dsRNA

Isolates of BTV exotic to the US were obtained from the Inter-American Bluetongue Project (Mo et al., 1994) and from the Onderstepoort Veterinary Institute virus library. The United States BTV and EHDV isolates were obtained from the Arthropod-Borne Animal Diseases Research Laboratory (ABADRL) reference collection. Table 1 lists the passage histories and virus strains used in this study. Sequences obtained from the literature are referenced. The dsRNA template was prepared using a differential lithium chloride precipitation procedure as described previously (Wilson et al., 1990). The S7 from selected strains of BTV serotypes 1, 3, 4, 6, 8 and 12 isolated from the Caribbean Basin and serotypes 1, 2, 3 and 4 isolated from South Africa were sequenced. In addition, two virus strains of BTV serotype 2 with distinct dsRNA electrophoretic patterns designated as Ona A and Ona B isolated from Ona, Florida (Collisson et al., 1985) were sequenced. For comparisons between related orbiviruses, the sequence of S7 from the prototype North American strain of EHDV serotype 2 was also determined.

2.2. Cloning and sequencing

Viral strains were cloned by reverse transcriptase/polymerase chain reaction (RT/PCR) using terminal primers as described previously (Wilson, 1994). Sequence data were obtained using standard automated sequencing (Smith et al., 1986; Applied Biosystems Inc., Foster City, CA) directly from PCR products and from cloned am-

Table 1
List of virus strains and Gen Bank accession numbers for L7 sequences

Reference number	Country	Year	Passage history[a]	Accession number	Reference[b]
BTV1 AU	Australia			M63417	Eaton et al., 1991
BTV1 SA	South Africa			X53740	Wade-Evans, 1990
BTV1 558 SA	South Africa	1977[c]	2 ECE, 5 BHK	AF188669	
BTV1 2172 Hond	Honduras	1989	1 ECE, 4 BHK	AF188670	
BTV2 US	USA			M64997	Kowalik and Li, 1991
BTV2 557 SA	South Africa	Unknown		AF188672	
BTV2 Ona A US	USA	1982	1 ECE, 2 BHK, 7 L929, 5 BHK	AF188674	
BTV2 OnaB US	USA	1982	1 ECE, 2 BHK, 7 L929, 2 BHK	AF188660	
BTV3 565 SA	South Africa	1977[c]	1 ECE, 7 BHK	AF188649	
BTV3 2030 Jama	Jamaica	1988	1 ECE, 3 BHK	AF188648	
BTV3 2034 Barb	Barbados	1988	1 ECE, 3 BHK	AF188644	
BTV3 2045 Toba	Tobago	1988	1 ECE, 6 BHK	AF188652	
BTV3 2058 CR	Costa Rica	1988	1 ECE, 3 BHK	AF188651	
BTV3 2131 Jama	Jamaica	1989	1 ECE, 6 BHK	AF188661	
BTV3 2152 Pana	Panama	1989	1 ECE, 3 BHK	AF188656	
BTV3 2154 Hond	Honduras	1989	1 ECE, 3 BHK	AF188646	
BTV3 2165 Trin	Trinidad	1989	1 ECE, 3 BHK	AF188647	
BTV3 2168 Pana	Panama	1989	1 ECE, 3 BHK	AF188657	
BTV3 2230 Guat	Guatemala	1990	1 ECE, 3 BHK	AF188659	
BTV3 2270 ES	El Salvador	1990	1 ECE, 4 BHK	AF188645	
BTV3 2343 Pana	Panama	1991	1 ECE, 3 BHK	AF188658	
BTV3 2350 Guat	Guatemala	1991	1 ECE, 3 BHK	AF188663	
BTV3 2354 CR	Costa Rica	1992	1 ECE, 3 BHK	AF188655	
BTV3 2364 Pana	Panama	1992	1 ECE, 4 BHK	AF188654	
BTV4 2227 DR	Dominican Republic	1990	1 ECE, 3 BHK	AF188667	
BTV4 566 SA	South Africa	1979[c]	1 ECE, 7 BHK	AF188668	
BTV6 2187 Hond	Honduras	1990	1 ECE, 3 BHK	AF188653	
BTV6 2233 Hond	Honduras	1990	1 ECE, 3 BHK	AF188662	
BTV8 2215 DR	Dominican Republic	1990	1 ECE, 3 BHK	AF188671	
BTV10 US	USA			X06463	Yu et al., 1988
BTV11 US	USA			M32102	Kowalik et al., 1990b
BTV12 2016 Jama	Jamaica	1988	1 ECE, 5 BHK	AF188673	
BTV13 US	USA			J04365	Kowalik and Li, 1989
BTV15 AU	Australia			L11724	Wang et al., 1994
BTV17 US	USA			X53693	Kowalik et al., 1990a
BTV17 240 Guat	Guatemala	1990	1 ECE, 6 BHK	AF188664	
BTV17 285 PR	Puerto Rico	1990	1 ECE, 6–7 BHK	AF188665	
BTV17 296 PR	Puerto Rico	1990	1 ECE, 6 BHK	AF188650	
BTV17 300 PR	Puerto Rico	1990	1 ECE, 6 BHK	AF188666	
EHDV1 NA	USA			D10766	Iwata et al., 1992
EHDV2 NA (SV 124)	USA	1973	4 Vero, 3 BHK	AF188643	

Table 1 (*Continued*)

Reference number	Country	Year	Passage history[a]	Accession number	Reference[b]
EHDV2 AU	Australia			EHU43560	Nagesha et al., unpublished
AHSV4				D12533	Roy et al., 1991
AHSV9				S69829	Wade-Evans et al., 1993

[a] Virus passage histories where known. The number prior to the abbreviation is the number of passages. Abbreviations: BHK, baby hamster kidney cells; ECE, embryonated chicken eggs; L929, mouse fibroblast cells; Vero, African green monkey kidney cells.
[b] Sequences not referenced are new S7 sequences reported in this paper.
[c] First recorded cell-culture passage.

plification products using the commercially available TA cloning kit (Invitrogen, San Diego, CA). Complete sequences were determined in both the sense and anti-sense directions. When plasmid clones were used to generate sequencing templates, three separate clones were sequenced to correct for any potential sequence errors in a single clone.

2.3. Computer analyses

Sequence data were compiled with SeqMan software (DNA Star Inc., Madison, WI) and analyzed with GCG software programs version 10 (Oxford Molecular Inc., Mountain View, CA). Phylogenetic analyses were performed using the following computer programs: Molecular Evolutionary Genetics Analysis, MEGA, version 1.01 (Kumar et al., 1993), Phylogeny Inference Package, PHYLIP version 3.5 (Felsenstein, 1993) and Phylogenetic Analysis Using Parsimony, PAUP version 4.0b3a (Swofford, 1997).

3. Results and discussion

The BTV S7 genes are 1154–1156 base pairs (bp) in length and have a single open reading frame encoding a predicted protein of 349 amino acids. The EHDV S7 gene is 1161 bp in length and also has a single open reading frame encoding a protein of 349 amino acids. Alignment of the deduced amino acid sequences demonstrates the conservation of this protein sequence. The more variable region that also contains the putative receptor-binding domain and has been shown to be surface accessible is depicted in Fig. 1. With the exception of BTV15 AU, the first 120 amino acid residues are very highly conserved (96.7–99.2% identity). This is probably a result of the amino and carboxy terminal residues involvement in the VP7-VP3 interactions in forming the core particle (Monastryskaya et al., 1997). Previously mapped immunodominant antigenic epitopes of the US serotypes at amino acid residues 122–139 and 11 residues at the carboxyl terminus (Li and Yang, 1990) are relatively conserved (Fig. 1, underlined; 82.4–88.2% and 81.8–90.9%, respectively). A BTV epitope at residues 263–267, that was previously identified as potentially cross-reactive with a related EHDV (Du Plessis et al., 1994), is highly conserved between the BTV serotypes (78.6–100%), EHDV serotypes (100%) and between BTV and EHDV serogroups (64.3%). However, these amino acid sequences are different in another orbivirus, AHSV.

The crystal structure of BTV10 VP7 has been reported and the upper domain is an anti-parallel β-sandwich and the strands common to the jelly-roll motif (Grimes et al., 1995). These common strands, as with the 263–267 epitope, are highly conserved among BTV strains and fairly conserved among EHDV strains. The RGD tripeptide (double underline) responsible for the integrin-dependent cell adhesion processes (Grimes et al., 1995) is conserved among all isolates except for BTV13 US and BTV15 AU. This position for BTV13 US has an isoleucine at the arginine position, which still maintains an integrin-dependent domain sequence. The lysine

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                                                    ####  ## ##             ####240
BTV1 558   DPMMIYLVWR RIENFAMAQG NSQQTQA... GVTVSVGGVD MRAGRIIAWD GQAALHVHNP
BTV1 SA    ---------- ---------- ---------- ---------- ---------- -------R--
BTV1 2172  ---------- ---------- ---------- ---------- ---------- ----------
BTV17 300  ---------- ---------- ---------- ---------- ---------- ----------
BTV17 US   ---------- ---------- ---------- ---------- ---------- ----------
BTV2 US    ---------- ---------- ---------- ---------- ---------- ----------
BTV10 US   ---------- ---------- ---------- ---------- ---------- ----------
BTV11 US   ---------- ---------- ---------- ---------- ---------- ----------
BTV1 2227  ---------- ---------- ---------- ---------- ---------- ----------
BTV2 OnaB  ---------- ---------- ---------- ---------- ---------- ----------
BTV3 2131  ---------- ---------- ---------- ---------- ---------- ----------
BTV6 2233  ---------- ---------- ---------- ---------- ---------- ----------
BTV3 2350  ---------- ---------- ---------- ---------- ---------- ----------
BTV4 566   ---------- ---------- ---------- ---------- ---------- ----------
BTV17 240  ---------- ---------- ---------- ---------- ---------- ----------
BTV17 285  ---------- ---------- ---------- ---------- ---------- ----------
BTV8 2215  ---------- ---------- ---------- ---------- ---------- ----------
BTV1 AU    ---------- ---------- -----L---- ---------- ---------- -----QI---
BTV2 557   ---------- -------P-- -----L---- ---------- ---------- -----QI---
BTV12 CB   ---------- -------P-- -----L---- ---------- ---------- -----QI---
BTV2 OnaA  ---------- -------P-- -----L---- ---------- ---------- -----QI---
BTV3 2154  ---------- -----S-P-- ---R-L---- ---------N ---------- ---V-QI---
BTV13 US   ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV3 2165  ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV3 2030  ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV6 2187  ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV3 2034  ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV3 2230  ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV3 2151  ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV3 SA    ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV17 296  ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV3 2364  ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV3 2270  ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV3 2058  ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV3 2168  ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV3 2354  ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV3 2343  ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV3 2045  ---------- -----S-P-- ---R-L---- ---------- ---------- ---V-QI---
BTV15 AU   ------II-- ---I--N--- --V------- -----I---N I-----T--- -----N----
EHDV1 NA   --V---F--- --GT-SN-A- -A-D-PQ--- ---LN----N ----V---Y- ---PVN-N--
EHDV2 NA   --I---F--- --GT-SN-A- -A-D-PQ--- ---LD----N ----V-V-Y- ---PVN-N--
EHDV2 AU   --I---F--- --GT-SS-A- -A-E-PQ--- ---LN----N ----V-V-Y- .--PVN-N--
AHSV4      -AV---F--- PLRI-CDP-- A-LESAPGAP -TF-T-D--N VA--DVV--N TI-PVN-G--
AHSV9      -AV---F--- PLRI-CDP-- A-LESAP--- -TF-T-D--N VA--DVV--N TI-PVN-G--
```

Fig. 1. Orbivirus partial deduced amino acid sequence alignment using standard single letter amino acid code. The region corresponds to the 120–240 amino acid position in the BTV alignment and surrounds the RGD putative integrin binding site (double underlined). Sequences are ordered based on the VP7 phylogenetic analysis. Cysteine residues are marked with an asterisk. A mapped epitope is underlined (Li and Yang, 1990) and residues accessible to the surface are marked with a hash marks (Eaton et al., 1991).

found to be critical for inner core structure formation (Le Blois and Roy, 1993) is conserved among all BTV and EHDV serotypes.

Genotypes specific to geographic location were not as clearly defined by phylogenetic analyses based on the complete S7 nucleic acid sequences (Fig. 2) as described previously for the L3 gene (Pritchard et al., 1995). For example, the US strains are primarily in the clade 1 but BTV13 US is in clade 5 containing strains isolated from Central America and the Caribbean Basin. As noted with L3 (Pritchard et al., 1995), the S7 sequences from South African BTV1 strains are more closely related to a strain of BTV1 from

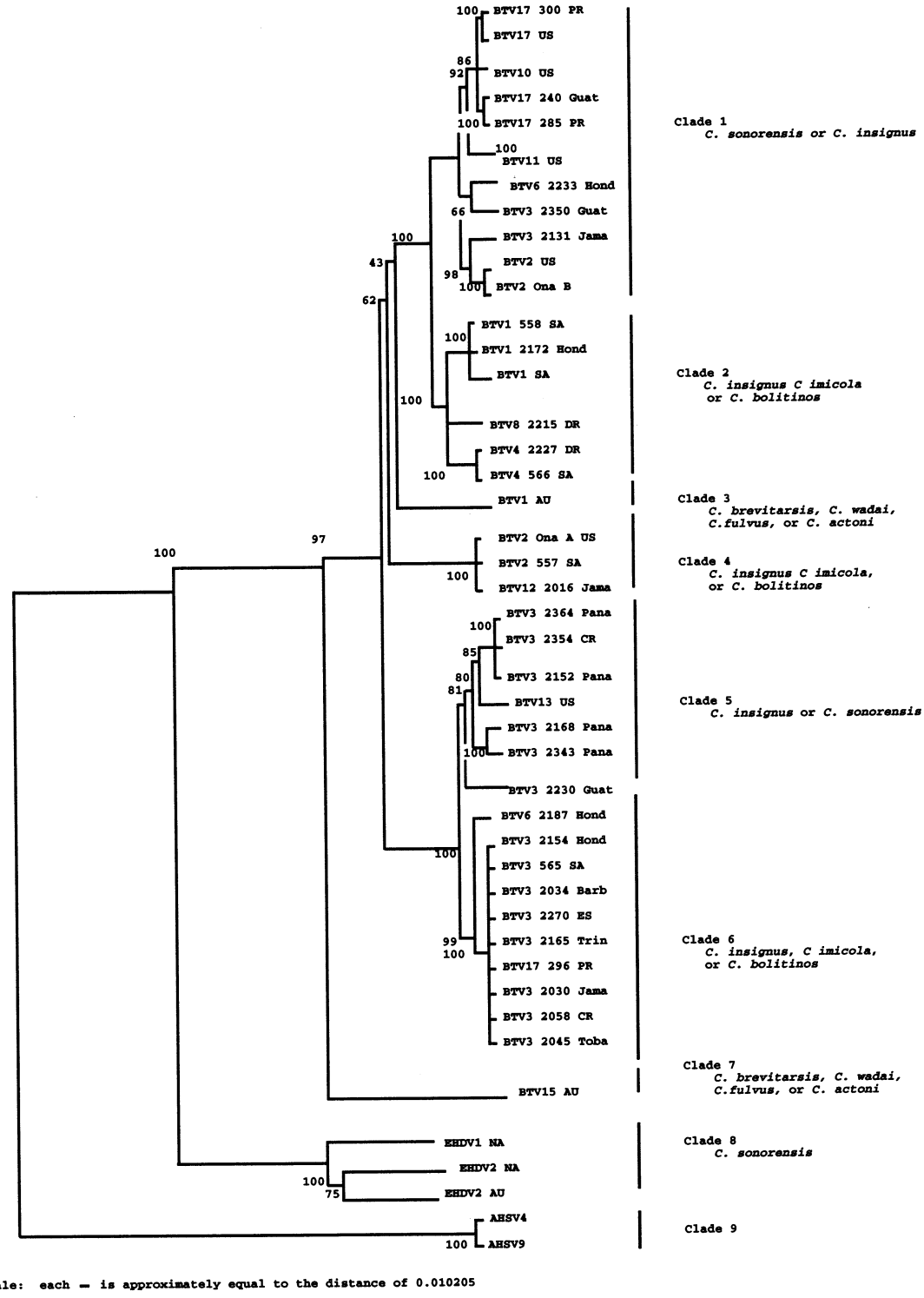

Fig. 2. Phylogenetic analysis of the S7 gene sequence for various orbiviruses. Potential vector species based on isolate origin are listed under clade or clade designation. The numbers indicate bootstrap confidence values after 500 replications. The tree was generated using MEGA and the following analysis parameters: gamma distance measure ($a = 1$), Kimura 2-parameter model and neighbor-joining bootstrap analysis.

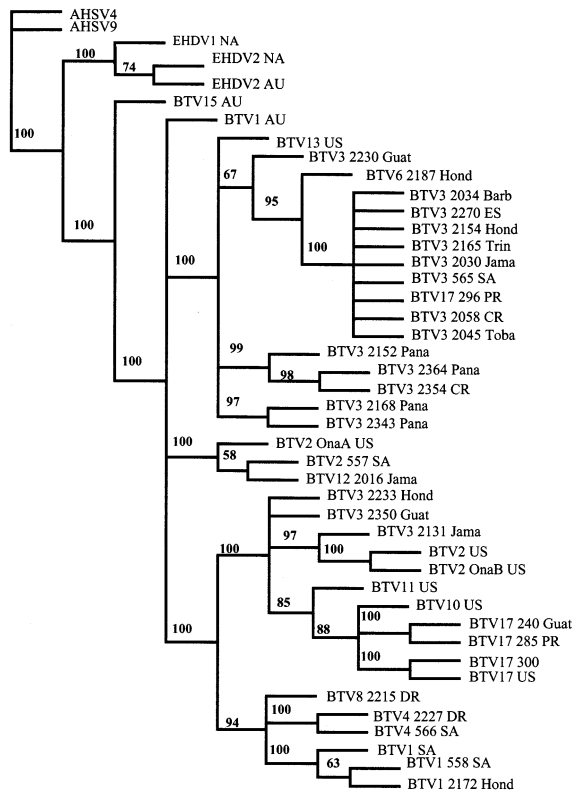

Fig. 3. A transition: transversion (3:1) weighted maximum parsimony analysis of the orbivirus S7 coding region using PAUP (Swofford, 1997). The numbers indicate bootstrap support values after 100 replications. The graphical tree representation was generated using treeview (Page, 1996).

Honduras than to the Australian BTV1 strain. The Australian BTV15 remains the most distinct serotype (Pritchard et al., 1995). Fig. 2 was generated using the Kimura two parameter model that assumes that all nucleotide substitutions occur randomly and that transitions are generally more frequent than transversions. The tree was generated using the neighbor-joining method that finds neighbors sequentially to minimize the total length of the tree. Maximum parsimony analysis generates trees that minimize the number of evolutionary changes (Li, 1997). The phylogenetic groupings are similar when the S7 coding region is analyzed with the maximum parsimony method (Fig. 3).

Amino acids that comprise the VP7 top domain (Monastryskaya et al., 1997) are likely involved in interactions of the virus with its receptor. Phylogenetic trees based on the nucleotide coding for this region identified the same genetic relations at similar confidence levels (data not shown). The rates of non-synonymous (K_a) and synonymous (K_s) changes were calculated (Li, 1993; data not shown). The K_a, as expected, was less than the K_s and the trends were similar to that depicted by the phylogenetic analysis. The K_a/K_s data can be used to demonstrate positive selection, however, in this case the results are inconclusive.

Three serotypes are thought to be recent introductions into geographic regions and representative strains of these serotypes were chosen for this study. The most recent serotype to be introduced in the US is BTV2. Early after its introduction, this serotype had an RNA electrophoretic profile similar to BTV2 from South Africa but quickly changed to an new profile that persisted during the outbreak (Collisson et al., 1985). The virus strains with the original profile are called BTV2 OnaA US and the strains with subsequent profile are called BTV2 OnaB US. The coding region S7 of BTV2 OnaA US was determined to be 99.8% identical to BTV2 557 SA and only 79.4% identical to BTV11 US. BTV2 OnaB US, however, was only 79.8% identical to BTV2 557 SA but 92.8% identical to BTV11 US. This data is consistent with the hypothesis that this virus originated from South Africa. This dramatic genetic change could be a result of a reassortment event with indigenous BTV strains or environmental selection of a more favorable genotype. Further study is needed to determine if there is a difference in the infection rate of *C. sonorensis* by these two virus types.

Bluetongue virus serotype 3 was the most recent serotype to be isolated in the Caribbean Basin and Central American region (Mo et al., 1994). The first S7 sequence determined from a BTV3 strain in this region was similar to the BTV3 565 SA sequence. This suggested that BTV3 in this region originated from South Africa. The S7 from several BTV3 strains were sequenced to determine if a genetic pattern of change could be followed as this serotype apparently moved across the region. Also, several strains isolated from Panama were sequenced to determine change over time. The early strains isolated in

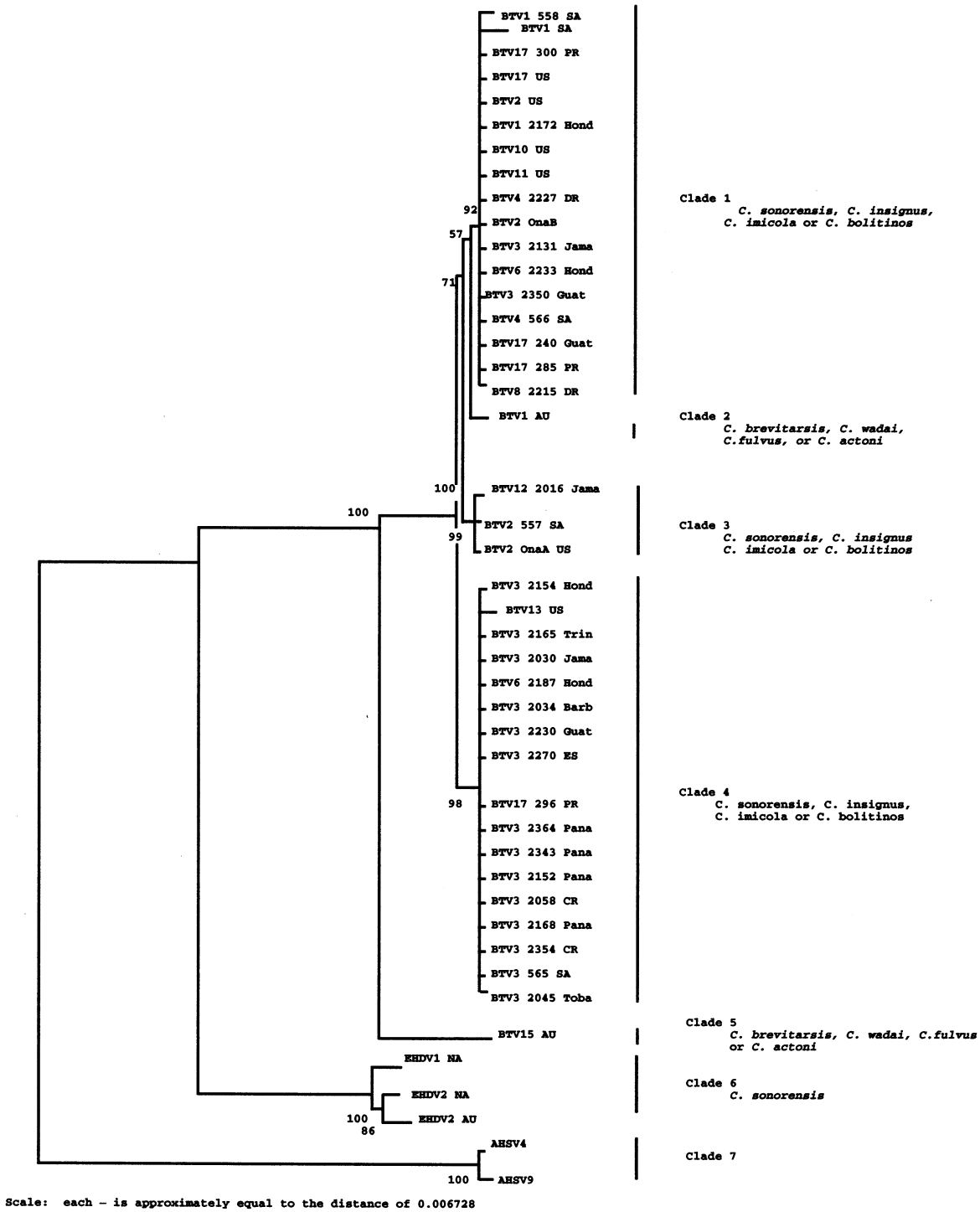

Fig. 4. Phylogenetic analysis of the deduced amino acid sequence for various orbiviruses. Potential vector species based on isolate origin listed under clade or clade designation. The tree was generated using MEGA and the following analysis parameters: Poisson correction and neighbor-joining bootstrap analysis. The numbers indicate bootstrap confidence values after 500 replications.

1988 were all in the nucleic acid phylogenetic clade 6 that included BTV3 565 SA. Two of the five 1989 strains were in clade 6, two in clade 5 and one in clade 1, containing the majority of US strains. The majority of the 1990–1992 BTV3 strains isolated in this region were in clades 5 and 1, with only one, BTV3 2270 ES, remaining in clade 6. Although only a small number of strains were examined, there was a general shift toward a S7 genotype similar to that found in the region.

It is suspected that the BTV17 isolated in Central America and the Caribbean Basin probably came from the US (Mo et al., 1994). The S7 sequence data from a few strains of BTV17 is supportive of this suspicion, as three of the four strains examined were phylogenetically in clade 1, which contains a majority of viruses of US origin. The outlier, BTV17 296 PR, was in clade 6 and may have resulted from reassortment. Alternatively, this virus could also represent a genetic shift towards a S7 genotype similar to that found in the region. Additional isolates after 1992 are not available to determine if these trends continued.

Phylogenetic grouping utilizing deduced amino acid sequences shows a reduction in the distinction of geographic clades (Fig. 4). These findings indicate there is random variation among virus populations resulting in at least five BTV VP7 types that are moderately dependent on geographical location. Four of the five US serotypes are in VP7 clade 1 and BTV13 is found in VP7 clade 4. The BTV13 prototype strain also contained the most distinct VP3 sequence among the US serotypes (Pritchard et al., 1995). The South African, Central American, and Caribbean Basin strains are spread between three VP7 clades. The most diverse are the two published Australian strains that separate into distinct clades. Perhaps the multiple *Culicoides* species associated with BTV transmission in Australia allow more variation in the VP7 protein. The South African *Culicoides* species are different from the Central America and Caribbean Basin principle species; however, viruses isolated within these regions are similar. This suggests that the virus-insect receptor interactions are similar.

The correlation between *Culicoides* species and viruses from particular S7 or VP7 clades is not strong. Neither phylogenetic trees generated using VP7 amino acid sequences in close proximity to the putative RGD integrin binding site nor trees generated using only the VP7 amino acid sequences thought to be accessible to the outer surface (120 residues) strengthened this correlation (data not shown). The lack of relationship may in part be due to use of prototype and cell-culture adapted virus isolates in this study. The lowest passage cell culture-adapted virus strains available were used because the original virus infected tissues were not available for direct PCR. Virus isolation through embryonated chicken egg inoculation (ECE) and cell-culture could select for a particular virus population. Still, differences in infection rates of colonized *C. sonorensis* have been shown with laboratory BTV US serotype strains (Mecham and Nunamaker, 1994). Also, differences in the infection rates of three Culicoides species by three South African serotypes have been demonstrated (Venter et al., 1998). The mechanistic controls for these differences are likely to be complex. Very little is known about the biochemical requirements for orbivirus infection of its invertebrate host. To understand this interaction, the genetics of the virus and the insect vector populations present needs to be considered. This study begins to define the genetics of one gene that is a component of the complex combination of factors involved in the infection process.

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